Formation of water-soluble metal cyanide complexes from solid minerals by *Pseudomonas plecoglossicida*

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Abstract

A few *Pseudomonas* species are able to form hydrocyanic acid (HCN), particularly when grown under glycine-rich conditions. In the presence of metals, cyanide can form water-soluble metal complexes of high chemical stability. We studied the possibility to mobilize metals as cyanide complexes from solid minerals using HCN-forming microorganisms. *Pseudomonas plecoglossicida* was cultivated in the presence of copper- and nickel-containing solid minerals. On powdered elemental nickel, fast HCN generation within the first 12 h of incubation was observed and water-soluble tetracyanaonickelate was formed. Cuprite, tenorite, chrysocolla, malachite, bornite, turquoise, millerite, pentlandite as well as shredded electronic scrap was also subjected to a biological treatment. Maximum concentrations of cyanide-complexed copper corresponded to a solubilization of 42% and 27% when *P. plecoglossicida* was grown in the presence of cuprite or tenorite, respectively. Crystal system, metal oxidation state and mineral hydrophobicity might have a significant influence on metal mobilization. However, it was not possible to allocate metal mobilization to a single mineral property. Cyanide-complexed gold was detected during growth on manually cut circuit boards. Maximum dicyanoaurate concentration corresponded to a 68.5% dissolution of the total gold added. These findings represent a novel type of microbial mobilization of nickel and copper from solid minerals based on the ability of certain microbes to form HCN.

Introduction

Only a few pseudomonads have been described as being cyanogenic, i.e. able to form hydrocyanic acid (HCN), especially when grown under glycine-rich conditions. In particular, *Pseudomonas aeruginosa* (Meganathan & Castric, 1977; Pessi & Haas, 2000), *Pseudomonas fluorescens* (Astrom, 1991; Faramarzi *et al*., 2004), *Pseudomonas putida* (Flaishman *et al*., 1996), and *Pseudomonas syringae* (Kremer & Souissi, 2001) are capable of generating HCN by oxidative decarboxylation from direct precursors such as glycine, glutamate, or methionine (Castric, 1977).

Pseudomonas plecoglossicida is known as the causative agent of a lethal fish disease occurring in ayu fish (*Plecoglossus altivelis*) (Nishimori *et al*., 2000). The organism was originally isolated from internal organs of dead fishes, but also occurs on the skin and fins (Sukenda, 2001). Besides fish, *P. plecoglossicida* can also be found in waste water, soil, sewage sludges, or in the roots of sand dune plants as an endophytic organism, especially in the wild rye (*Elymus mollis*) (Song *et al*., 2003; Chowdhury *et al*., 2004; Ekhaise, 2004; Park *et al*., 2005). From a more physiological point of view, *P. plecoglossicida* has been also reported to produce siderophores under iron-limited growth conditions (Meyer *et al*., 2002). Interestingly to note is that the strain is also able to grow in solutions of 5% NaCl (Nishimori *et al*., 2000). Until today, *P. plecoglossicida* is not known as being a cyanogenic organism.

Generally, cyanide is formed as secondary metabolite during the early stationary growth phase (Knowles & Bunch, 1986). Cyanide occurs in solution as free cyanide which includes the cyanide anion (CN^-) and the nondissociated HCN. At physiological pH, cyanide is present mainly as HCN because of its pK_a value of 9.3 and is, therefore, volatile. In the presence of salts however, this value decreases to *c*. 8.3 and the volatility is reduced (Fagan, 1998). In addition, in many cases (particularly in microbial growth media or under natural conditions), cyanide is complexed

by cyanicidic compounds ('cyanide killers') which reduce the volatility again. Cyanicides include carbonic acids, humic acids, sulfate, arsenic, arsenate, iron, oxidized forms of zinc, and antimony (Fagan, 1998).

From a chemical point of view, cyanide can interact with a series of metals. It is known that nearly all transitions metals (except lanthanides and actinides) form well-defined cyanides complexes which show a very good water solubility and a very high chemical stability (Chadwick & Sharpe, 1966; Barnes *et al*., 2000). By combining microbiological and chemical principles, namely 'microbiological cyanide formation' and 'chemical metal complexation by cyanide,' we report here the ability of *P. plecoglossicida* to form HCN resulting in metal mobilization from solid materials. The objectives of the work were (i) to investigate growth and HCN generation by *P. plecoglossicida* under various growth conditions (varying glycine concentration, initial pH, or amount of solids added); and (ii) to study the formation of water-soluble metal cyanides when the organism is grown in the presence of metal-containing solid materials. Very recently, we have reported the ability of HCN-generating microorganisms (*P. fluorescens, Chromobacterium violaceum, Bacillus megaterium*) to form water-soluble nickel and gold cyanides when exposed to nickel powder or shredded electronic waste (Faramarzi *et al*., 2004).

Until today, the microbially mediated formation of water-soluble cyanide complexes from solid materials has been considered only marginally. Few reports describe the bacterial solublization of gold by *C. violaceum* from gold-containing ore or coupons of pure gold and the subsequent formation of gold cyanide (Smith & Hunt, 1985; Lawson *et al*., 1999; Campbell *et al*., 2001).

Materials and methods

Different *Pseudomonas* strains were isolated from soil collected in Tehran (main campus of Tehran University of Medical Sciences, Iran) on cetrimide agar which is selective for the growth of pseudomonads. Samples from top soil were suspended in cetrimide broth and plated on cetrimide agar. For screening purposes, ability of HCN generation was checked using a qualitative colorimetic spot test (Feigl & Anger, 1966). After isolation, identification was performed by the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strain was assigned to *Pseudomonas plecoglossicida* with a similarity of 99.1% based on morphology, motility, utilization of carbon sources, and cellular fatty acid composition.

Cells were routinely cultured for maintenance in 250 mL baffled Erlenmeyer flasks in 50 mL of cetrimide medium containing (in $g\,L^{-1}$) cetrimide (0.2); gelatine peptone (20.0); casein hydrolysate (10.0); magnesium chloride (1.4); and potassium sulfate (10.0); glycerol (10 mL). pH was 7.3 ± 0.2. Long-term storage of the organisms was carried out in 15% glycerol at $-80\,^{\circ}C$. Luria–Bertani broth containing tryptone (10.0), yeast extract (5.0), sodium chloride (10.0) was used for growth experiments. The pH was adjusted to 7.2 except when experiments regarding different initial pH values were performed. Glycine and metal-containing solids were additionally supplemented in different amounts according to the experimental setup. Duplicate cultures were grown in 250 mL baffled Erlenmeyer flasks in 100 mL of medium and incubated at $30\,^{\circ}C$ on a rotary shaker at 150 r.p.m. Bacterial growth was monitored by determining the optical density at 450 nm. The pH was recorded additionally. For metal mobilization experiments, different amounts (up to $10\,g\,L^{-1}$) of solid materials (e.g. nickel, different copper minerals, shredded electronic scrap) were added to the medium.

Copper- and nickel-containing mineral and ore samples (chrysocolla, malachite, bornite, millerite, pentlandite, bunsenite) were received from the collection of the Geological Institute of the ETH (Zurich, Switzerland). Elemental nickel (which served as a model compound), cuprite, tenorite, and turquoise were obtained commercially. All minerals were manually crushed (except nickel which was already obtained as powder) and sorted to remove the host rock. Remaining solids were ground to powder using a ball mill and sieved to obtain particles $< 71\,\mu m$.

Free cyanide was quantitatively analyzed applying the picric acid colorimetric method (Drochioiu *et al*., 2003). Analyses of metal complexed cyanides were performed by reversed phase high pressure liquid chromatography (rP-HPLC) (Faramarzi *et al*., 2004). Metal-complexed cyanides were separated at $40\,^{\circ}C$ on a hydrophobic C-18 column. The eluent consisted of 25% acetonitrile; 150 mM orthophosphoric acid; 60 mM tetrabutylammonium hydroxide (TBAOH); and 2.34 mM sodium perchlorate. pH was adjusted with sodium hydroxide to 7.3. Flow rate was set at $1\,mL\,min^{-1}$. Metal cyanides were determined by UV detection at 229, 230, and 267 nm for Au, Cu, and Ni, respectively. Commercially available corresponding metal cyanides were used as standards.

Results and discussion

Growing *Pseudomonas plecoglossicida* on additional glycine ($1\,g\,L^{-1}$) in the medium, a fast formation of HCN within the first 12 h of incubation was observed (Fig. 1a). However, as compared with *P. fluorescens* and *Chromobacterium violaceum* (Faramarzi *et al*., 2004), maximum cyanide concentrations were much lower (*c*. by a factor of 50). In parallel to growth, water-soluble tetracyanaonickelate, $[Ni(CN)_4]^{2-}$, was formed from powdered elemental nickel which served as a model compound (Fig. 1b). Growth (as determined by

OD and pH) was not influenced by the addition of nickel powder.

To enhance tetracyanonickelate formation, different amounts of glycine and nickel powder were added to the growth medium. In addition, initial pH was varied. In the presence of 1 g nickel per liter, increasing amounts of glycine gradually reduced growth as determined by OD (Fig. 2a). Maximum tetracyanonickelate concentration was obtained at a glycine concentration of 5 g L^{-1}. As HCN is directly produced from glycine (Wissing, 1968), optimal glycine concentrations were determined for the cultivation of *P. plecoglossicida* in the presence of 1 g nickel powder per liter (Fig. 2a). Concentrations of 3–7 g glycine per liter resulted in the highest tetracyanonickelate concentrations. Increased glycine concentrations (>7 g L^{-1}) led to reduced growth as well as reduced tetracyanonickelate formation.

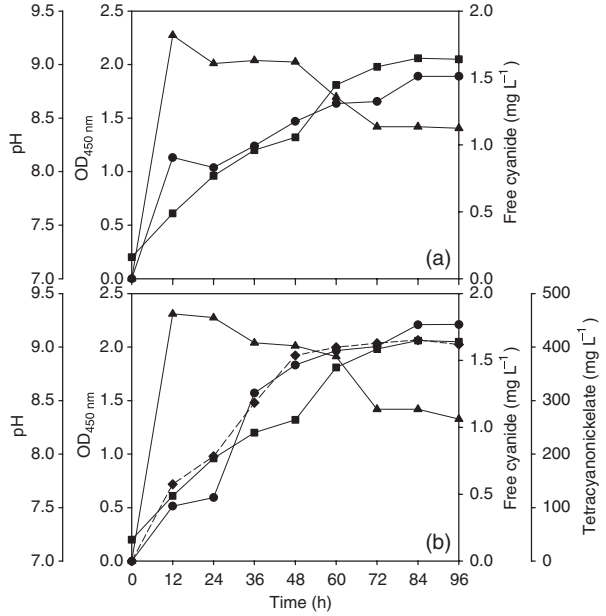

Fig. 1. Growth, generation of cyanide, and formation of tetracyanonickelate by *Pseudomonas plecoglossicida* in Luria–Bertani medium. (a) Supplemented with glycine (1 g L^{-1}); (b) supplemented with glycine (1 g L^{-1}) and powdered elemental nickel (1 g L^{-1}). ●, OD at 450 nm; ■, pH; ▲, free cyanide; ◆, tetracyanonickelate.

The addition of nickel powder stimulated growth to a certain extent (Fig. 2b). Maximum OD was obtained at nickel concentrations of *c.* 4 g L^{-1}. However, at concentrations > 4 g L^{-1} growth was drastically reduced. The reasons might be either toxic effects of nickel and/or mechanical stress owing to increased pulp densities. Pulp density effects have already been observed during growth of *C. violaceum* and *P. fluorescens* in the presence of nickel powder (Faramarzi *et al.*, 2004). As known from other reports, growth of *Acidithiobacillus* species in the presence of pyrite or covellite was also dependent on pulp density (Curutchet *et al.*, 1990; Baldi *et al.*, 1992). Regarding the formation of tetracyanonickelate by *P. plecoglossicida*, optimal concentrations of solid nickel were between 1 and 3 g L^{-1} (Fig. 2b). Although growth was more or less constant over an initial pH range of 6 to 9.5 as determined by OD measurement, a steady decrease in tetracyanonickelate concentration was observed (Fig. 2c). Maximum tetracyanonickelate concentration was obtained at initial pH of 6.5.

In addition to powdered nickel, various copper, and nickel minerals were also subjected to a biological treatment in suspensions of 0.2 g L^{-1} (Table 1). Regarding copper oxides, maximum cyanide-complexed copper concentration corresponded to a solubilization of 42% and 27% when grown in the presence of cuprite (Cu_2O) and tenorite (CuO), respectively. Crystal system, metal oxidation state as well as mineral surface hydrophilicity might have a

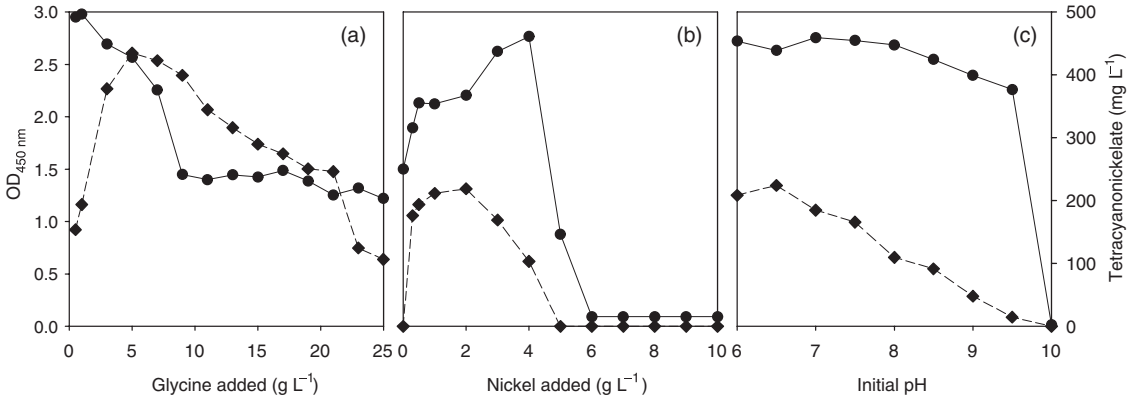

Fig. 2. Growth and tetracyanonickelate formation by *Pseudomonas plecoglossicida* in Luria–Bertani medium. (a) Supplemented with different of amounts of glycine and powdered nickel (1 g L^{-1}); (b) supplemented with different amounts of powdered nickel and glycine (1 g L^{-1}); (c) as function of different initial pH values on powdered nickel (1 g L^{-1}) and glycine (1 g L^{-1}). Measurements were taken after an incubation of 48 h. ●, OD at 450 nm; ◆, tetracyanonickelate.

Table 1. Mobilization of metal as corresponding cyanide complexes from different copper- and nickel-containing minerals by *Pseudomonas plecoglossicida*

Element	Mineral	Formula	Metal oxidation state	Dana class*	Crystal system*	Crystal class*	Hydrophilicity†	Metal mobilization (%)‡
Copper	Cuprite	Cu_2O	+1	4.1	Isometric	Hexoctahedral	B	42.0
	Tenorite	CuO	+2	4.2	Monoclinic	Prismatic	B	27.2
	Chrysocolla	$(Cu,Al)_2H_2Si_2O_5(OH)_4 \cdot n(H_2O)$	+2	74	Orthorhombic	Pyramidal	F	23.4
	Malachite	$Cu(CO_3)(OH)_2$	+2	16a	Monoclinic	Prismatic	D	8.9
	Bornite	Cu_5FeS_4	+1	2.5	Orthorhombic	Dipyramidal	B	5.0
	Turquoise	$CuAl(PO_4)_4(OH)_8$	+1	42	Triclinic	Pinacoidal	D	tr§
Nickel	Nickel	Ni	0	1	Isometric	Hexoctahedral	B	5.5
	Millerite	NiS	+2	2.8	Trigonal	Hexagonal scalenohedral	B	1.4
	Pentlandite	$Fe_{4.5}Ni_{4.5}S_8$	+2	2.7	Isometric	Hexoctahedral	B	0.14
	Bunsenite	NiO	+2	4.2	Isometric	Hexoctahedral	E	tr§

*According to Dana's New Mineralogy (Gaines *et al*., 1997).

†According to Wakamatsu (1997) with F being the group with the highest hydrophilicity.

‡Maximum after 36–48 h of incubation as percentage of the initial amount of copper or nickel added (200 mg solids per liter).

§tr, traces.

significant influence on metal mobilization (Table 1). However, it was not possible to allocate metal mobilization to a single mineral trait. It has been reported for the oxidation of sulfidic minerals by *Acidithiobacillus* that the mineral structure is of major importance for the metal mobilization from solids (Sand *et al*., 2001). This might also be the case for the metal solubilization by cyanogenic microorganisms.

In addition to solid minerals, we have also investigated the potential of *P. plecoglossicida* to mobilize metals from solid waste such as scrap from electronic equipment. Residues from the mechanical recycling of used electronic equipment (e.g. computers) represent a highly complex metal-containing matrix (Brandl *et al*., 2001). These solid wastes often contain metals with a high economic value. Particularly gold is of special interest and can occur in concentrations of $20\,mg\,kg^{-1}$ shredded printed circuit boards. Gold-containing pieces ($5 \times 10\,mm$) of printed circuit boards were used for growth experiments. These were obtained by manually cutting printed circuit boards followed by manual sorting. After a lag phase of *c.* 36 h, cyanide-complexed gold (dicyanoaurate, $[Au(CN)_2]^-$) was detected in the culture fluid (Fig. 3). Maximum dicyanoaurate concentration corresponded to a 68.5% dissolution of the gold added. In this respect, *P. plecoglossicida* proved to be more efficient than *C. violaceum* under identical growth conditions (Faramarzi *et al*., 2004).

As being part of the soil microbiota (Kremer & Souissi, 2001; Benizri *et al*., 2005) and its close association with plant roots (Park *et al*., 2005), one might speculate about the ecological role of cyanogenic microorganisms such as *P. plecoglossicida* in soil. From an ecological viewpoint, it is

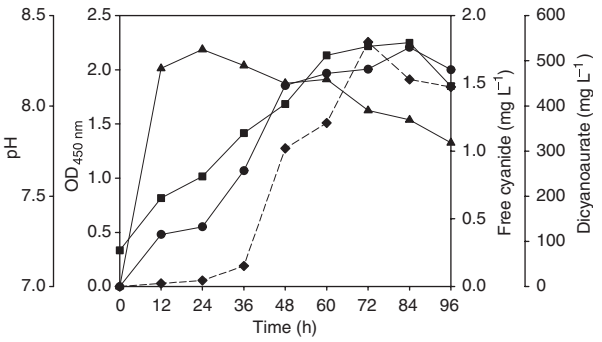

Fig. 3. Growth, generation of cyanide, and formation of dicyanoaurate by *Pseudomonas plecoglossicida* in Luria–Bertani medium supplemented with glycine ($1\,g\,L^{-1}$) and with gold-containing pieces of shredded circuit boards (resulting in *c.* 500 mg Au per liter). ●, OD at 450 nm; ■, pH; ▲, free cyanide; ◆, dicyanoaurate.

assumed that HCN formation has an advantage for the organism by inhibiting competing microorganisms (Blumer & Haas, 2000). It has been demonstrated that HCN formed in the rhizosphere (predominantly by pseudomonads) adversely affected plant growth because of growth inhibition of seedlings (Kremer & Souissi, 2001). Besides its influence on plant growth, cyanide formed by *P. aeruginosa* has been demonstrated as being an agent which rapidly paralyzes and kills the nematode *Caenorhabditis elegans*. This might represent a defensive mechanism against grazing (Gallagher & Manoil, 2001). However, besides controlling agent to inhibit competitors, microbially formed cyanide might also act in soil environments as lixiviant for metal compounds which can be subsequently been taken up by plants and

microorganisms. We have demonstrated that *P. plecoglossicida* is able to mobilize nickel and copper from the corresponding solids which might also be the case in soil under *in situ* conditions. Until today, there are no comprehensive reports on this hypothesis despite the known presence of cyanogens in the rhizosphere. In addition, cyanogenic microorganisms might also find an industrial application regarding solid waste treatment for the recovery of metals. We have shown that *P. plecoglossicida* mobilizes gold when grown in the presence of electronic scrap.

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